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Immune and anti-oxidant effects of *in ovo* selenium proteinate on post-hatch experimental avian necrotic enteritis[☆]



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ABSTRACT

This study was conducted to investigate the effects of *in ovo* administration of selenium (Se) incorporated into hydrolyzed soybean protein (B-Taxim [BT]) on protection against experimental avian necrotic enteritis (NE). Broiler eggs were injected with either 100 µl of PBS alone (BT0), or 20 or 40 µg/egg of BT in PBS (BT20, BT40) at 18 days of embryogenesis. On day 14 post-hatch, the chickens were uninjected or orally infected with 1.0×10^4 oocysts of *Eimeria maxima* (*E. maxima*). On day 18 post-hatch, *E. maxima*-infected chickens were orally infected with 1.0×10^9 CFU of *Clostridium perfringens* (*C. perfringens*). Compared with untreated and infected BT0 controls, BT20 and/or BT40 birds showed increased body weights, decreased fecal shedding of *E. maxima* oocysts, lower serum α-toxin and NetB levels, increased levels of serum antibodies against *C. perfringens* α-toxin and NetB toxin, decreased levels of serum malondialdehyde, reduced serum catalase and superoxide dismutase catalytic activities, and increased intestinal levels of gene transcripts encoding interleukin (IL)-1β, IL-6, IL-8, and peroxiredoxin-6, but decreased levels of transcripts for catalase and glutathione peroxidase. Interestingly, transcript levels for inducible nitric oxide synthase and paraoxonase/arylesterase 2 were decreased in the BT20 group and increased in the BT40 group, compared with BT0 controls. These results indicate that *in ovo* administration of broiler chickens with a Se-containing protein hydrolysate enhanced protection against experimental NE possibly by altering the expression of proinflammatory and anti-oxidant genes and their downstream pathways.

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1. Introduction

Selenium (Se) is an micronutrient for both human and veterinary medicine. Published studies have suggested that dietary Se deficiency alters cytokine and chemokine expression during microbial infections (Beck and Matthews, 2000). Se deficiency is associated with impaired immune responses to infectious agents and reduced anti-oxidant enzyme activities (Smith et al., 2011; Wang et al., 2009a). In mammals, dietary Se supplementation protected against bacterial endotoxin-induced oxidative stress and regulated cytotoxic T lymphocyte and natural killer cell activities (Sakaguchi et al., 2000; Kiremidjian-Schumacher et al., 1994). An association between altered dietary Se levels and the incidence of infectious diseases has also been described in poultry (Colnago et al., 1984; Larsen et al., 1997; Mahmoud and Edens, 2005; Gabrashanska et al., 2007).

Reactive oxygen species (ROS) induce lipid peroxidation to generate malondialdehyde (MDA), an *in vivo* biomarker of oxidative stress (Del Rio et al., 2005; Stancliffe et al., 2011). Superoxide is a major intracellular ROS whose levels are controlled by superoxide dismutase (SOD), a key anti-oxidant enzyme that prevents superoxide from reacting with critical cellular targets. A phytonutrient, *Agaricus brasiliensis* polysaccharide, inhibited lipid peroxidation and significantly lowered increased SOD activity in a rat model of reperfusion injury (Zhang et al., 2010). In Se-deficient mice or chickens, MDA levels in blood were increased, and SOD, catalase (CAT), and glutathione peroxidase 7 (GPx7) levels were increased compared with feeding a normal Se diet (Wang et al., 2009a; Peng et al., 2012). Conversely, dietary Se supplementation decreased MDA content, improved hepatic CAT and GPx7 activities, and regulated cytokine gene expression in mice infected with *Listeria monocytogenes*, compared with normal Se controls (Wang et al., 2009a).

Necrotic enteritis (NE) and avian coccidiosis are important infectious diseases in commercial poultry production, both resulting in field outbreaks with substantial mortality (Lee et al., 2011a,b,c; Van Immerseel et al., 2009). NE alone costs the international poultry industry approximately 2 billion U.S. dollars annually as a consequence of medication and reduced body weight (Cooper et al., 2009; Van der Sluis, 2000). Both enteric diseases have traditionally been controlled by prophylactic in-feed antibiotics (Shirley and Lillehoj, 2012). The incidence of *Clostridium perfringens*-associated NE has increased worldwide in the last decade primarily due to decreased use of antibiotic growth promoters and antimicrobials. Recent interest, therefore, has focused on the development drug-free disease control strategies (Seal et al., 2013). Dietary supplementation with and *in ovo* administration of natural nutrients and phytochemicals have been reported to enhance post-hatch poultry growth, promote early gut development, improve immune status, and prevent pathogen infections (Foye et al., 2007; Lee et al., 2010, 2013; Tako et al., 2005). While dietary Se supplementation of chickens has been shown to enhance protective immunity to some enteric pathogens (Colnago et al., 1984; Larsen et al., 1997; Mahmoud and Edens, 2005; Gabrashanska et al., 2007), the potential of

in ovo Se in reducing negative effects of avian NE has not been investigated yet. Therefore, the current study was undertaken to evaluate the effects of a commercial Se supplement, B-Taxim Se, on protective immunity against experimental avian NE and to correlate this effect with markers of gut inflammation and anti-oxidant enzymes.

2. Materials and methods

2.1. B-Taxim Se

B-Taxim Se (BT) (Pancosma S.A., Geneva, Switzerland) was formed by the incorporation of inorganic Se within soybean protein that is subsequently hydrolyzed to give a commercial product specifically designed for animal feeding. BT contains 1.1% (wt/wt) Se metal content.

2.2. *In ovo* injection

All experiments were approved by the USDA, Agricultural Research Service Institutional Animal Care and Use Committee (IACUC). Embryonated eggs of inbred broiler chickens (Moyer's Chicks, Inc., Quakertown, PA) were incubated at 37.5 °C with a relative humidity of 55–60% for 18 days and candled to select well-developed embryos. At 18 days, eggs were injected with 100 µl of sterile phosphate buffered saline (PBS, pH 7.4) alone (BT0), or 20 or 40 µg/egg of BT in PBS (BT20, BT40) using an Intelliject system (Avitech, Easton, MD). The doses of BT were chosen based on unpublished, preliminary results demonstrating significant anti-oxidant and immune effects without apparent toxicity. Briefly, each egg was cleaned and positioned in a holder under an injection needle with the large end on top. With the help of a vacuum system, a disinfected needle penetrated the shell past the air cell to deliver the inoculum into the amniotic cavity. After each inoculation, the needle was disinfected to minimize the risk of infection. In addition, the system was designed to avoid negative pressure inside of the egg, thus reducing the risk of cross-contamination (Lee et al., 2010). Following *in ovo* injection, the eggs were incubated at 36 °C with 60–65% relative humidity until hatch. Hatched chickens were continuously fed *ad libitum* with a basal diet containing 0.1 mg/kg Se for 20 days and were maintained in a temperature-controlled environment at 30 °C for the first 2 days of growth, followed by a gradual reduction in the temperature to 23 °C until end of the experiment. Se concentrations in BT solutions and the basal diet were analyzed as described (AOAC, 2000) using an inductively coupled plasma-mass spectrometry (ICP-MS) system (7500 series, Agilent, Santa Clara, CA). Powder samples were dissolved by microwave digestion (MicroPrep Q2000, Questron Technologies Corp., Mississauga, Ontario, Canada) using a mixture of hydrofluoric, nitric, and perchloric acids (Merck, Whitehouse Station, NJ). Se concentrations were determined using external calibration curves.

2.3. NE disease model

Chickens (20/group) were kept in brooder pens in an *Eimeria*-free facility for 14 days post-hatch and transferred

into large hanging cages (2 birds/cage) at a separate location where they were infected with *Eimeria maxima* and *C. perfringens* and kept until the end of the experimental period. To facilitate the development of NE, birds were fed an antibiotic-free certified organic starter diet (USDA/FeedMill, Beltsville, MD) containing 17% crude protein and 61% carbohydrate between days 1 and 18 post-hatch and a standard grower diet containing 24% crude protein and 54% carbohydrate between days 18 and 24 post-hatch. Chickens were uninfected or orally infected on day 14 with *E. maxima* strain 41A (1.0×10^4 oocysts/bird) and on day 18 with *C. perfringens* strain Del-1 (1.0×10^9 colony forming units [CFU]/bird) as described (Jang et al., 2012; Lee et al., 2012, 2013; Park et al., 2008). The Del-1 strain has been used in the NE disease model and expresses the α -toxin and NetB toxin (Lee et al., 2013).

2.4. Body weights, gut lesion scores, and fecal oocyst shedding

Body weights ($n=20$ /group) were measured on days 0 and 14 (prior to *E. maxima* infection), and on day 20 (day 6 post-infection with *E. maxima* and day 2 post-infection with *C. perfringens*) (Lee et al., 2011b, 2013). For gut lesions, birds ($n=5$ /group) were euthanized and sacrificed by cervical dislocation at day 2 post-infection with *C. perfringens*. Two equal intestinal sections of 10 cm located anterior and posterior to the diverticulum were collected and scored on a scale of 0 (none) to 4 (high) in a blinded fashion by three independent observers as described (Lee et al., 2013). For the determination of fecal oocyst shedding, birds ($n=15$ /group) were placed in oocyst collection cages and fecal samples were collected between days 19 and 24 as described (Lee et al., 2012). Oocyst numbers were determined using a McMaster chamber according to the formula: total oocysts per bird = oocyst count \times dilution factor \times (fecal sample volume/counting chamber volume)/2.

2.5. Serum α -toxin and NetB toxin levels and antibodies to α -toxin and NetB toxin

Blood samples ($n=5$ birds/group) were collected by cardiac puncture immediately following euthanasia at days 2 and 6 post-infection with *C. perfringens* to determine the levels of α -toxin or NetB toxin in serum and also to measure the serum Ab levels against α -toxin or NetB toxin. All serum samples were prepared by centrifugation at 3000 rpm for 10 min at 4 °C to remove blood clots.

To measure serum toxin levels, antigen capture assay method which we described previously was used (Lee et al., 2013, 2014). Capture mAb detecting α -toxin or NetB toxin which were developed by ARS-USDA were coated at 0.5 µg/well in 96 well ELISA plates overnight at 4°C. The plates were blocked and washed, and ELISA was carried out as previously described (Lee et al., 2013, 2014). Chicken sera were diluted 1:2 in PBS-T, 100 µl added to the wells and incubated for 2 h at room temperature. The plates were washed with PBS-T, and 100 µl/well of peroxidase-conjugated anti- α -toxin or NetB toxin detection antibodies were added

and incubated for 30 min followed by development with 3,3',5,5'-tetramethylbenzidine substrate as described (Lee et al., 2013, 2014). Optical densities at 450 nm were measured and serum α -toxin or NetB toxin concentrations were determined using a standard curve generated with known concentrations of each purified recombinant protein.

Serum IgG antibody levels against two enterotoxins, α -toxin and NetB toxin, were measured by an indirect enzyme-linked immunosorbent assay (ELISA) as described (Lee et al., 2013; Jang et al., 2012). Ninety-six well microtiter plates were coated overnight with 1.0 µg/well of purified recombinant α -toxin or NetB toxin proteins which were expressed in *Escherichia coli* as previously described (Lee et al., 2012). The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 1% bovine serum albumin. Serum samples were diluted 1:100, 100 µl was added to each well, incubated with agitation for 2 h at room temperature, and washed with PBS-T. Bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgG secondary antibody and tetramethylbenzidine substrate (Sigma, St. Louis, MO). Optical densities (OD) were measured using a microplate spectrophotometer (ELx800™, BioTek, Winooski VT). Each data in the infected groups expresses OD value after subtracting OD of the baseline data obtained from non-treated and uninfected control broiler chickens.

2.6. MDA levels, and CAT and SOD activities

Blood samples ($n=5$ birds/group) were collected by cardiac puncture immediately following euthanasia at day 2 post-infection with *C. perfringens* for determination of serum MDA levels, and CAT and SOD activities. Serum MDA levels were measured using a commercial kit (Life Science Specialties, Vancouver, WA). Serum CAT and SOD activities were measured using the Catalase Activity Assay Kit (Cell Biolabs, San Diego, CA) and SOD determination kit (Sigma), respectively.

2.7. Cytokine/chemokine and anti-oxidant gene transcript levels

On day 2 post-infection with *C. perfringens*, 20-cm segments of the intestinal jejunum located proximal to the Meckel's diverticulum were collected (Lee et al., 2013) using 5 birds per group. The jejunum was cut open longitudinally, gently washed with ice-cold Hank's Balanced Salt Solution (Sigma), and the mucosal layer was carefully removed using a cell scraper. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 µl of 10× reaction buffer (Sigma) and incubated for 15 min at room temperature. One microliter of stop solution was added to inactivate DNase I, and the mixture was heated for 10 min at 70 °C. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Quantitative RT-PCR oligonucleotide primers for chicken IL-1 β , IL-6, IL-8, TNFSF15, inducible nitric oxide synthase (iNOS), paraoxonase 2 (PON2), CAT, SOD,

Table 1

Oligonucleotide primers used for quantitative RT-PCR.

Transcript	Primer	Primer sequence	PCR product size (bp)	GenBank accession no.
GAPDH	Forward	5'-GGTGGTGCTAACCGTGTAT-3'	264	NM_204305.1
	Reverse	5'-ACCTCTGTCACTCTCCACA-3'		
IL-1 β	Forward	5'-TGGGCATCAAGGGCTACA-3'	244	NM_204524.1
	Reverse	5'-TCGGTTGGTGGTGTATG-3'		
IL-6	Forward	5'-CAAGGTGACCGAGGAGGAC-3'	254	NM_204628.1
	Reverse	5'-TGGCGAGGAGGGATTCT-3'		
IL-8	Forward	5'-GGCTTGCTAGGGAAATGA-3'	200	NM_205498.1
	Reverse	5'-AGCTGACTCTGACTAGGAAACTGT-3'		
TNSSF15	Forward	5'-CCTGAGTATTCCAGCAACGCA-3'	292	NM_01024578
	Reverse	5'-ATCCACCACTTGATGTCACAA-3'		
iNOS	Forward	5'-TGGGTGGAAGGCCAAATA-3'	241	U46504
	Reverse	5'-GTACCAAGCCCTTGAAGGAC-3'		
PON2	Forward	5'-ACGTGACAGACACATCCAT-3'	5592	NM_001201468.1
	Reverse	5'-CGTGAGCCAGAACACTGAAA-3'		
CAT	Forward	5'-ACTGCAAGGCCAAAGTGT-3'	864	NM_001031215.1
	Reverse	5'-GGCTATGGATGAAGGATGGA-3'		
SOD	Forward	5'-ATTACCGGCTTGTCTGATGG-3'	666	NM_205064.1
	Reverse	5'-CCTCCCTTGCACTCACATT-3'		
GPx7	Forward	5'-TCACCACTTCAGAATGCAG-3'	223	NM_001163245.1
	Reverse	5'-TCCAACACTGGAAATTCTG-3'		
PRDX6	Forward	5'-TTACAGAAGGATCTCCCATC-3'	1276	NM_001039329.2
	Reverse	5'-GGATGTGGAGGATTGGCTTA-3'		

glutathione peroxidase 7 (GPx7), peroxiredoxin 6 (PRDX6), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control, are listed in Table 1. Amplification and detection were carried out with the Mx3000P system and Brilliant SYBR Green qPCR master mix (Stratagene). The reverse transcription product was diluted to 1:10, and 5 μ l was used for PCR amplification. PCR conditions were as follows: denaturation at 95 °C for 10 min followed by amplification at 72 °C for 1 min for 40 cycles. Standard curves were generated using log₁₀ diluted standard RNA to determine amplification efficiency and the levels of individual transcripts were normalized to those of GAPDH by the Q-gene program (Lee et al., 2013). Each sample was analyzed in triplicate. To normalize individual replicates, the logarithmic-scaled threshold cycle (C_t) values were transformed to linear units of normalized expression prior to calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression using the Q-gene program.

2.8. Statistical analysis

Each sample was analyzed in quadruplicate. All data were subjected to one-way analysis of variance using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL) and were expressed as mean \pm SEM. Mean values of treatment groups were compared using the Tukey's multiple comparison test and differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effect of in ovo BT on body weights, intestinal lesions, and fecal oocyst shedding

BT injection did not affect embryo viability, hatchability, or post-hatch sex ratio, and did not exhibit gross pathologic effects when used at the denoted concentrations (20

or 40 μ g/egg). No significant differences in body weights of chickens treated with BT0, BT20, or BT40 were seen on days 0 and 14 post-hatch and prior to infection (data not shown). However, on day 20 post-hatch, chickens treated with BT20 or BT40 and co-infected with *E. maxima* and *C. perfringens* exhibited increased body weights compared with untreated and infected controls (BT0) (Fig. 1A). Indeed, body weight among the BT20 and BT40 groups were equal to those of the untreated and uninfected control group (Cont) (Fig. 1A). Birds treated *in ovo* with Se and co-infected with *E. maxima* and *C. perfringens* showed less depression and diarrhea, and reduced intestinal lesion scores of 2.2 and 2.1 in BT20 and BT40 groups, respectively, compared with 2.6 in the control BT0 ($P > 0.05$). However, there was no mortality in any group during the experiment. Birds which were treated with BT20 or BT40 and co-infected with *E. maxima* and *C. perfringens*, also showed significantly decreased fecal oocyst shedding compared with the BT0 group (Fig. 1B).

3.2. Effect of in ovo BT on serum α -toxin and NetB toxin levels and serum antibodies to α -toxin and NetB toxin

Levels of α -toxin and NetB toxin in serum increased in the NE infected groups (B0, B20, and B40) compared with the non-supplemented and non-infected control group. Feeding dietary Se (BT20 and BT40 groups) significantly reduced both toxin levels in serum in NE-afflicted chickens compared with the non-supplemented and infected controls (Table 2).

Since serum antibodies against *C. perfringens* α -toxin and NetB toxin have been suggested to play a protective role during experimental NE (Keyburn et al., 2010; Lee et al., 2012, 2013), we assessed antibodies against both enterotoxins in serum samples from untreated and BT-treated chickens following NE induction. Higher serum antibodies against α -toxin and NetB toxin were detected in the chickens afflicted with NE compared with the

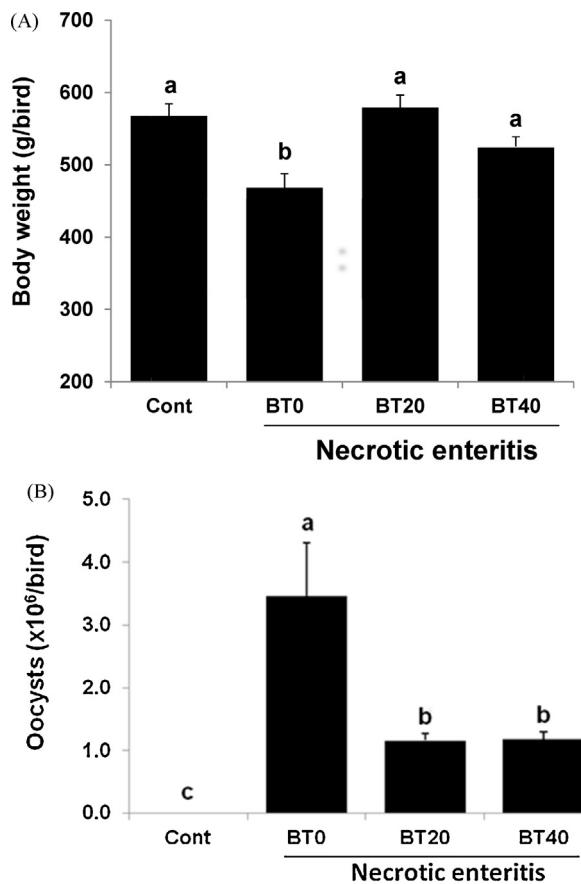


Fig. 1. Effect of *in ovo* BT treatment on body weights and fecal oocyst shedding. Broiler eggs were injected with 100 μ l of PBS alone (BT0), BT20, or BT40 as described in Section 2. On day 14 post-hatch, chickens were uninfected (Cont) or orally infected with 1.0×10^4 sporulated oocysts of *E. maxima*. On day 18 post hatch, *E. maxima*-infected chickens were orally infected with 1.0×10^9 CFU of *C. perfringens* to induce Necrotic enteritis. (A) Body weights were measured on day 2 post infection with *C. perfringens* ($n = 20$ birds/group). (B) Fecal oocysts were measured between days 19 and 24 post hatch. Each bar represents the mean \pm SEM value ($n = 15$ birds/group). Bars not sharing the same letters are significantly different according to the Tukey's multiple comparison test ($P < 0.05$).

non-supplemented and uninfected control. BT20 and BT40 groups showed higher serum antibodies against *C. perfringens* α -toxin and NetB toxin compared with the BT0 group (Table 2). However, the significant difference from BT0 in

the serum antibody levels against *C. perfringens* α -toxin and NetB toxin was shown in BT20 group.

3.3. Effect of *in ovo* BT on serum MDA levels, and CAT and SOD enzyme activities

A prior study demonstrated that a low Se diet increased the levels of MDA and decreased CAT activity in spleens of broiler chickens (Peng et al., 2012). Therefore, we investigated the effects of *in ovo* BT on these markers of oxidative stress. Serum MDA levels, and CAT and SOD activities were increased by 73.5%, 42.8%, and 3.0%, respectively, in untreated chickens following infection with *E. maxima* and *C. perfringens*, compared with untreated and uninfected controls (Fig. 2). However, infected chickens treated with BT20 or BT40 had significantly decreased levels of all three markers of oxidative stress, compared with the BT0 group. In the case of BT20, MDA levels and CAT activity were equal to those of the untreated and uninfected control group (Cont), and in the case of BT40 CAT activity was even lower than the Cont group.

3.4. Effect of *in ovo* BT on intestinal cytokine and anti-oxidant enzyme gene transcript levels

Given the suppressive effect of *in ovo* BT on CAT and SOD activities (Fig. 2), and the established protective role of chicken cytokines and other markers of inflammation and innate immunity during experimental NE (Jang et al., 2012; Lee et al., 2013), we next investigated the effect of *in ovo* BT on intestinal cytokine and anti-oxidant enzyme gene expression. Intestinal levels of transcripts encoding IL-1 β , IL-6, IL-8 and PRDX6 were increased in chickens treated with BT20 and/or BT40, and co-infected with *E. maxima* and *C. perfringens*, compared with the untreated and infected BT0 group (Table 3). In contrast, mRNAs encoding CAT, PON2, and GPx7 were decreased in the BT20 and/or BT40 groups, compared with the BT0 group. Other proinflammatory mediators were either unaffected by BT (TNFSF15, SOD) or showed a mixed response.

4. Discussion

This study was conducted to evaluate the effect of *in ovo* treatment with BT as a Se supplement on protection against experimental avian NE and NE-induced levels of proinflammatory and anti-oxidant mediators in the gut.

Table 2

Effect of *in ovo* BT on serum α -toxin and NetB toxin levels and serum antibodies against α -toxin and NetB toxin.

Groups	Toxin OD (ng/ml)		Ab titer OD	
	α -Toxin	NetB	α -Toxin	NetB
Control	0.08 \pm 0.01 (0.3 \pm 0.3) ^c	0.06 \pm 0.01 (0.1 \pm 0.1) ^c	0.17 \pm 0.01 ^c	0.25 \pm 0.02 ^c
BT0	0.47 \pm 0.01 (56.0 \pm 1.4) ^a	0.40 \pm 0.01 (73.9 \pm 1.4) ^a	0.33 \pm 0.01 ^b	0.47 \pm 0.02 ^b
BT20	0.35 \pm 0.02 (11.3 \pm 0.6) ^b	0.32 \pm 0.02 (12.8 \pm 0.6) ^b	0.55 \pm 0.02 ^a	0.65 \pm 0.01 ^a
BT40	0.36 \pm 0.02 (13.9 \pm 1.0) ^b	0.31 \pm 0.02 (11.6 \pm 1.0) ^b	0.41 \pm 0.03 ^b	0.54 \pm 0.02 ^b

Eggs were injected with PBS alone (Control, BT0), BT20, or BT40, and chickens were co-infected with *E. maxima* and *C. perfringens* post-hatch as described in Fig. 1. Blood samples from 5 birds/group were collected on day 2 post-infection with *C. perfringens* and the sera were used to measure the levels of α -toxin and NetB toxin by antigen capture ELISA. Serum antibody (Ab) levels to α -toxin or NetB toxin were measured by indirect ELISA on day 6 post-infection with *C. perfringens* ($n = 5$ birds/group) and each data in the infected groups were expressed as OD values. Each data represents the mean \pm SEM value ($n = 5$ birds/group). Within each column, values with different letters are significantly different according to the Tukey's multiple comparison test ($P < 0.05$).

Table 3Effect of *in ovo* BT on the levels of intestinal proinflammatory cytokine and anti-oxidant enzyme transcripts^A.

Transcript	Control	BT0	BT20	BT40
Necrotic enteritis				
IL-1 β	2.5E – 07 ± 4.1E – 08 ^a	3.2E – 07 ± 3.9E – 08 ^{ab}	6.7E – 07 ± 1.6E – 07 ^c	6.0E – 07 ± 1.2E – 07 ^{bc}
IL-6	0.08 ± 0.01 ^a	0.10 ± 0.01 ^a	0.15 ± 0.01 ^b	0.15 ± 0.01 ^b
IL-8	0.47 ± 0.02 ^a	0.50 ± 0.07 ^a	0.40 ± 0.05 ^a	0.70 ± 0.05 ^b
TNFSF15	3.8E – 04 ± 3.4E – 05 ^a	1.2E – 03 ± 2.1E – 04 ^b	9.7E – 04 ± 1.8E – 04 ^b	1.2E – 03 ± 7.1E – 05 ^b
iNOS	2.9E – 02 ± 3.3E – 04 ^{ab}	2.4E – 02 ± 2.2E – 03 ^b	1.7E – 02 ± 2.5E – 04 ^c	3.1E – 02 ± 2.3E – 03 ^a
PON2	4.4E – 11 ± 1.7E – 12 ^a	1.8E – 10 ± 4.8E – 12 ^b	1.2E – 10 ± 6.7E – 12 ^c	6.1E – 10 ± 2.2E – 11 ^d
CAT	0.025 ± 0.001 ^a	0.017 ± 0.006 ^a	0.006 ± 0.002 ^b	0.003 ± 0.000 ^b
SOD	0.06 ± 0.01 ^a	0.08 ± 0.00 ^a	0.07 ± 0.01 ^a	0.07 ± 0.01 ^a
GPx7	2.2E – 05 ± 8.4E – 06 ^{ab}	4.2E – 05 ± 1.9E – 05 ^a	2.3E – 06 ± 3.2E – 07 ^b	1.5E – 05 ± 6.0E – 06 ^{ab}
PRDX6	0.44 ± 0.01 ^{ab}	0.29 ± 0.03 ^c	0.36 ± 0.02 ^{bc}	0.48 ± 0.05 ^a

Eggs were injected with PBS alone (BT0), BT20, or BT40 and chickens were co-infected with *E. maxima* and *C. perfringens* as described in Section 2 using an established protocol. On day 2 post-infection with *C. perfringens*, 20-cm segments of the intestinal jejunum located proximal to the Meckel's diverticulum were used to measure intestinal proinflammatory cytokine and anti-oxidant enzyme transcript levels. Each data represents the mean ± SEM value ($n=5$ birds/group). Within each row, values with different letters are significantly different according to the Tukey's multiple comparison test ($P<0.05$).

^A Values represent mean ± SEM transcript levels normalized to the GAPDH internal control ($n=5$ birds/group).

Compared with untreated eggs, embryos treated with BT and co-infected post-hatch with *E. maxima* and *C. perfringens* showed (1) increased body weight, (2) reduced fecal oocyst levels, (3) decreased serum α -toxin and NetB toxin levels, (4) higher levels of serum antibodies against α -toxin and NetB toxin, (5) decreased levels of serum MDA, as well as CAT and SOD enzyme activities, (6) increased intestinal levels of mRNAs encoding proinflammatory cytokines, and (7) decreased levels of intestinal transcripts for CAT and GPx7 anti-oxidant enzymes. These collective results suggest that the *in ovo* administration of Se as the BT product has an overall protective effect against experimental NE in broiler chickens through its ability to modulate proinflammatory and anti-oxidant genes.

Both Se-treated groups (BT20 and BT40) exhibited greater body weights compared with the BT0 untreated control group, although there was no statistically significant difference between the two doses. Increased body weight in the BT-treated and infected groups may be attributed to improved intestinal physiology allowing for greater nutrient absorption, compared with the untreated and infected BT0 control group. Similar activities were proposed to account for the protective effect of dietary Se on protective immunity against *Eimeria* infection in broiler chickens (Colnago et al., 1984). Similarly, both the BT20 and BT40 groups showed significantly reduced fecal oocyst shedding, a major criteria of *Eimeria* infection, compared with the BT0 group. Furthermore, the groups treated with BT showed decreased serum α -toxin and NetB toxin levels. It may be explained by the increased levels of antibodies against *C. perfringens* α -toxin and NetB toxin in BT20 and BT40 (Lee et al., 2013). However, interestingly, the significant effect of BT treatment was found only in the BT20, but not in BT40 chickens when compared to the untreated BT0 group. This lack of an effect on antibody levels at the higher BT dose might be due to a concentration-dependent B cell apoptosis, as has been reported for other trace elements (Ibs and Rink, 2003).

MDA is a reactive aldehyde that forms covalent protein adducts, advanced lipoxidation end products, and its levels are often used as a biomarker of oxidative stress (Moore and Roberts, 1998; Del Rio et al., 2005; Farmer and Davoine,

2007). MDA is formed from polyunsaturated lipids by the action of ROS, including hydroxyl radical ($\cdot\text{OH}$), hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), and oxygen (O_2). ROS, in turn, are degraded by anti-oxidant enzymes, such that a reciprocal relationship exists between MDA levels and the levels/activities of anti-oxidants. CAT catalyzes the decomposition of H_2O_2 to O_2 and H_2O , while SOD catalyzes the dismutation of O_2^- into the same end products (Gaetani et al., 1996). Corroborating the observations of this study, Georgieva et al. (2007) reported increased MDA levels and decreased SOD activity in *Eimeria tenella*-infected chickens, compared with uninfected controls. Oxidative stress in mammals during infection by other members of apicomplexan parasites (*Toxoplasma gondii* and *Cryptosporidium parvum*) also has been associated with altered levels of MDA and/or anti-oxidant enzymes (Wang et al., 2009b; Engin et al., 2012).

In addition to CAT and SOD, a wide array of other anti-oxidant enzymes is responsible for maintaining normal redox homeostasis. Among these, the GPx family of multiple isozymes catalyzes the reduction of H_2O_2 or organic hydroperoxides to H_2O or the corresponding alcohols using reduced glutathione (GSH) as an electron donor ($\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GS} - \text{SG} + 2\text{H}_2\text{O}$) (Fink and Scandalios, 2002). GPx7 is a monomeric glutathione peroxidase localized to the endoplasmic reticulum. Similar to the diminution of CAT and SOD activities in serum following BT20 or BT40 treatment of *E. maxima/C. perfringens*-infected chickens, intestinal gene expression of GPx7 was reduced in the BT20 group compared with the BT0 group. The decreased serum MDA and antioxidant enzyme activities in both BT20 and BT40 groups denoted that Se supplementation clearly exerted a protective effect reducing oxidative stress induced by necrotic enteritis. These results agree with Monteiro et al. (2009) and Betancor et al. (2012) who also reported that Se supplementation induced lower MDA, CAT, and SOD activities. By contrast, intestinal expression levels of PRDX6 transcripts, which encodes a member of the thiol-specific anti-oxidant family of proteins, was higher in the BT40 group, but unchanged in the BT20 group, compared with the BT0 group. Similarly, PON2 gene transcripts were increased in the BT40

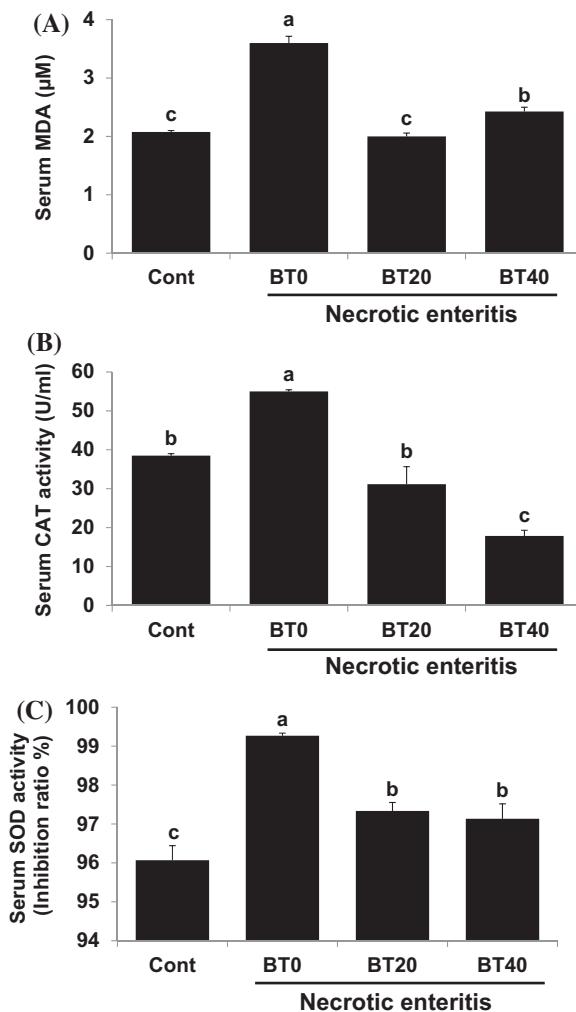


Fig. 2. Effect of *in ovo* BT on serum MDA levels, and CAT and SOD enzyme activities. Eggs were injected with PBS alone (BT0), BT20, or BT40 and chickens were uninfected (Cont) or co-infected with *E. maxima* and *C. perfringens* to induce necrotic enteritis as described in Fig. 1. Serum MDA levels, and CAT and SOD enzyme activities were measured on day 2 post infection with *C. perfringens*. Each bar represents the mean \pm SEM value ($n=5$ birds/group). Bars not sharing the same letters are significantly different according to the Tukey's multiple comparison test ($P<0.05$).

group, but reduced in the BT20 group, compared with BT0 controls. Part of the augmented response observed in the case of PON2 may be related to its known defense role in response to pathogenic microorganisms, in addition to its anti-oxidant activity (Teiber et al., 2008).

Increased levels of intestinal cytokines and chemokines play a critical protective role during experimental avian NE (Lee et al., 2011a,b,c, 2013). IL-1 β and IL-6 are a proinflammatory cytokines that regulates antibody and cell-mediated immune responses to *Eimeria* infections (Hong et al., 2006; Lee et al., 2010). IL-8 is a CXC chemokine that attracts leukocytes, primarily neutrophils, to mucosal sites of inflammation, while iNOS is induced by another proinflammatory cytokine, IFN- γ (Hong et al., 2006). High levels of the iNOS transcript were seen in the gut of *E. maxima*-infected chickens (Swaggerty et al., 2004). In

the current study, transcripts for the proinflammatory cytokines examined were increased either by BT20 alone (IL-1 β), BT40 alone (IL-8), or both BT20 and BT40 (IL-6). Future studies are needed to further test and compare the effects of different Se-containing compounds on experimental avian NE and other enteric diseases of importance to the commercial poultry industry.

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